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Modification of Operating Procedure for EZ-Retriever™ Microwave to Produce Consistent and Reproducible Immunohistochemical Results

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14. ABSTRACT
The present study was conducted to optimize the operating procedure for the EZ- Retriever™ microwave oven to produce consistent and reproducible staining results with microtubule-associated protein 2 (MAP-2). After performing microwave antigen retrieval (MAR) at 98°C, as recommended by the manufacturer, inconsistent patterns of MAP-2 immunoreactivity were produced. Uniform patterns of MAP-2 staining are critical when performing qualitative and quantitative image analysis on sections; therefore, optimizing the temperature at which MAR was performed was essential. Results indicate that when using the EZ-Retriever™ microwave, boiling sections in 10mM citric acid of pH 6.0 for 10 min at 106°C is optimal for recovering MAP-2 in formalin-fixed, paraffin-embedded sections. Ultimately, this modification will permit more accurate and reliable image analyses in future immunohistochemical studies, including other proteins that require citrate based solutions for MAR

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Abstract

The present study was conducted to optimize the operating procedure for the EZ- Retriever™ microwave oven to produce consistent and reproducible staining results with microtubule-associated protein 2 (MAP-2). After performing microwave antigen retrieval (MAR) at 98℃, as recommended by the manufacturer, inconsistent patterns of MAP-2 immunoreactivity were produced. Uniform patterns of MAP-2 staining are critical when performing qualitative and quantitative image analysis on sections; therefore, optimizing the temperature at which MAR was performed was essential. Results indicate that when using the EZ-Retriever™ microwave, boiling sections in 10mM citric acid of pH 6.0 for 10 min at 106℃ is optimal for recovering MAP-2 in formalin-fixed, paraffin-embedded sections. Ultimately, this modification will permit more accurate and reliable image analyses in future immunohistochemical studies, including other proteins that require citrate based solutions for MAR.

Introduction

Microwave antigen retrieval (MAR) is a technique frequently used to recover antigenic binding sites masked by formalin-fixation to produce markedly improved immunohistochemical staining. Previously, we established optimal antigen retrieval (AR) conditions for microtubule-associated protein 2 (MAP-2) using a Pelco 3440 Max microwave oven (Ted Pella, Inc., Redding, CA) (Pleva et al., 2002; Kan et al., 2005). AR of MAP-2 consisted of boiling sections in a 10mM citric acid solution of pH 6.0 for two 5-min cycles, with the power of the microwave oven set at 100%. However, due to the age of the microwave oven and the wattage of the oven, the temperature of the retrieval solution could not be controlled. Therefore, prior to placing sections in the oven, the AR solution was first brought to a continuous boil. After placing the sections in solution, each 5-min cycle was broken into two equal time periods of 2.5 min so that more solution could be added to compensate for loss due to boiling over and to avoid drying out the sections.

Recently, our laboratory acquired an EZ-Retriever^{TM} microwave oven (BioGenex, San Ramon, CA), designed specifically for AR of formalin-fixed, paraffin-embedded tissue sections. The microwave has a built-in temperature probe to measure solution temperature and automatically regulates heat intensity. In addition, the slide baths and slide holders supplied with the microwave are made of heat stable polymers to help eliminate the potential of solutions boiling away.

According to the manufacturer's instructions (EZ-Retriever^{TM} Microwave Operating Manual Version 1.0), the temperature can be set between 40 and 110° C; however, setting the temperature parameter higher than 98°C is not recommended when filling the tanks with EZ-AR3 or EZ-AR4 (BioGenex, San Ramon, CA), both citra-based solutions. Therefore, initial MAR trials were executed using citric acid solution heated at 98°C to retrieve MAP-2 antigenic binding sites. However, after performing MAP-2 immunohistochemistry, staining results were undesirable (Figure 1). MAP-2 immunostaining was not uniform throughout the section, rendering it unsuitable for reliable interpretation. The present experiment was undertaken to optimize use of the EZ-Retriever^{TM} microwave oven for MAR by resetting the temperature of the microwave oven.

Methods

2.1 Tissue Preparation

Routinely formalin-fixed (10% neutral phosphate buffered formalin for 24 hr) rat brain sections were cut coronally in a rat brain matrix (ASI Instruments, Warren, MI). Brain samples were processed in paraffin, sectioned serially at 5 μm, and mounted on positively charged slides (Fisher Scientific, Pittsburgh, PA). Brain sections were allowed to dry at room temperature for 24 hr and then processed for MAR immunohistochemistry.

2.2 Microwave Pretreatment

Sections were dewaxed in xylene, hydrated to distilled water, and then incubated in 5% hydrogen peroxide for 15 min at room temperature to suppress endogenous peroxidase activity. Following thorough washing in running tap water (5 min), sections were rinsed in distilled water. Ten mM (.21g/100ml) citric acid monohydrate (Sigma-Aldrich; St Louis, MO; Lot 30H-0627), adjusted to pH 6.0 by adding 2N NaOH, while monitoring with a pH meter (Beckman Instruments, Fullerton, CA), was used as an antigen retrieval solution. The probe tank, which contains a slot for the temperature control probe, was filled with 200-250 ml of the citric acid

antigen retrieval solution. Sections were placed into a slide basket and submerged in the tank. Another microwave tank was filled with an equivalent volume of water, and the two tanks were placed in opposing positions in the tank holder, according to the manufacturer's instructions (EZ-Retriever™ Microwave Operating Manual Version 1.0). Using the touch screen interface, 2 cycles of 5 min were selected, and the temperature of each cycle was set between 98°C and 110°C. After completion of the first cycle, the tanks were removed from the microwave oven, filled with fresh solution, and the second cycle was started. Following the second 5-min cycle of boiling in the microwave for a total time of 10 min, sections were allowed to cool at room temperature for a minimum of 20 min prior to processing for MAP-2 immunohistochemistry.

2.3 MAP-2 Immunohistochemistry

Indirect MAP-2 immunohistochemistry was performed using the avidin-biotin-peroxidase complex (ABC) method of Hsu et al. (1981). Brain sections, rinsed twice in phosphate buffered saline (PBS), pH 7.4 (Sigma Diagnostics, St. Louis, MO; Lot 082K6049), were incubated in 5% normal horse serum for 30 min at 4°C to block non-specific binding sites of tissue immunoglobulins to secondary antibody. Sections were then incubated with mouse monoclonal MAP 2a,b,c Ab-3 antibody (Lab Vision Corporation, Fremont, CA) for 18 hr at 4°C. Following two washes with PBS, sections were incubated with biotinylated secondary antibody (1:200 dilution) (Vector, Burlingame, CA) for 1 hr at room temperature, washed twice with PBS, and allowed to react with ABC solution (Vector, Burlingame, CA) for 30 min at room temperature. The presence of MAP-2 immunoreactivity was visualized as a brown precipitate after incubating sections in DAB-H_2O_2 solution (Sigma-Fast DAB tablet sects, Sigma-Aldrich, St. Louis, MO) for 5 min. The tablet sets were dissolved in 6 ml instead of 5 ml as recommended by the manufacturer, a modification made to attenuate the rate of DAB-H_2O_2 reaction. Finally, sections were coverslipped with Permount® (Fisher Scientific, Fair Lawn, NJ) for light microscopic examination. Overall intensity of MAP-2 was determined by computer assisted image analysis using Image-Pro® Plus software (Media Cybernetics, Silver Spring, MD).

Results

Results are summarized in Figures 2-5. The specificity, intensity, and uniformity of MAP-2 immunostaining were highly influenced by the temperature at which MAR was performed. Following MAR at 98°C, MAP-2 staining was uneven in the cortex (Figure 2A and Figure 3A) and in the hippocampus (Figure 4A and Figure 5A). In addition, MAP-2 staining of apical dendrites of pyramidal neurons in the stratum radiatum layer of the CA3 region of the hippocampus appeared fragmented (Figure 5A). Higher ranges of temperature were tested and 106°C was the minimum temperature at which a continuous boil was produced. Typical patterns of MAP-2 staining were observed in the cortex (Figure 2B and Figure 3B) and in the hippocampus (Figure 4B and Figure 5B) of sections that were boiled at 106°C. MAP-2 staining was homogenous, with specific and intense immunoreactivity in neuronal soma, apical dendrites, and finer dendritic processes (Figure 3B and Figure 5B).

Computer-assisted image analysis confirmed insufficient MAP-2 immunoreactivity following MAR at 98°C. Density, indicative of intensity of MAP-2 labeling, was 9.6×10^8, while density of MAP-2 staining following MAR at 106°C was 1.10877×10^9. In addition, the percentage of the area of the brain labeled with MAP-2 after MAR at 98°C was 29.2%, compared to 33.1% after MAR at 106°C.

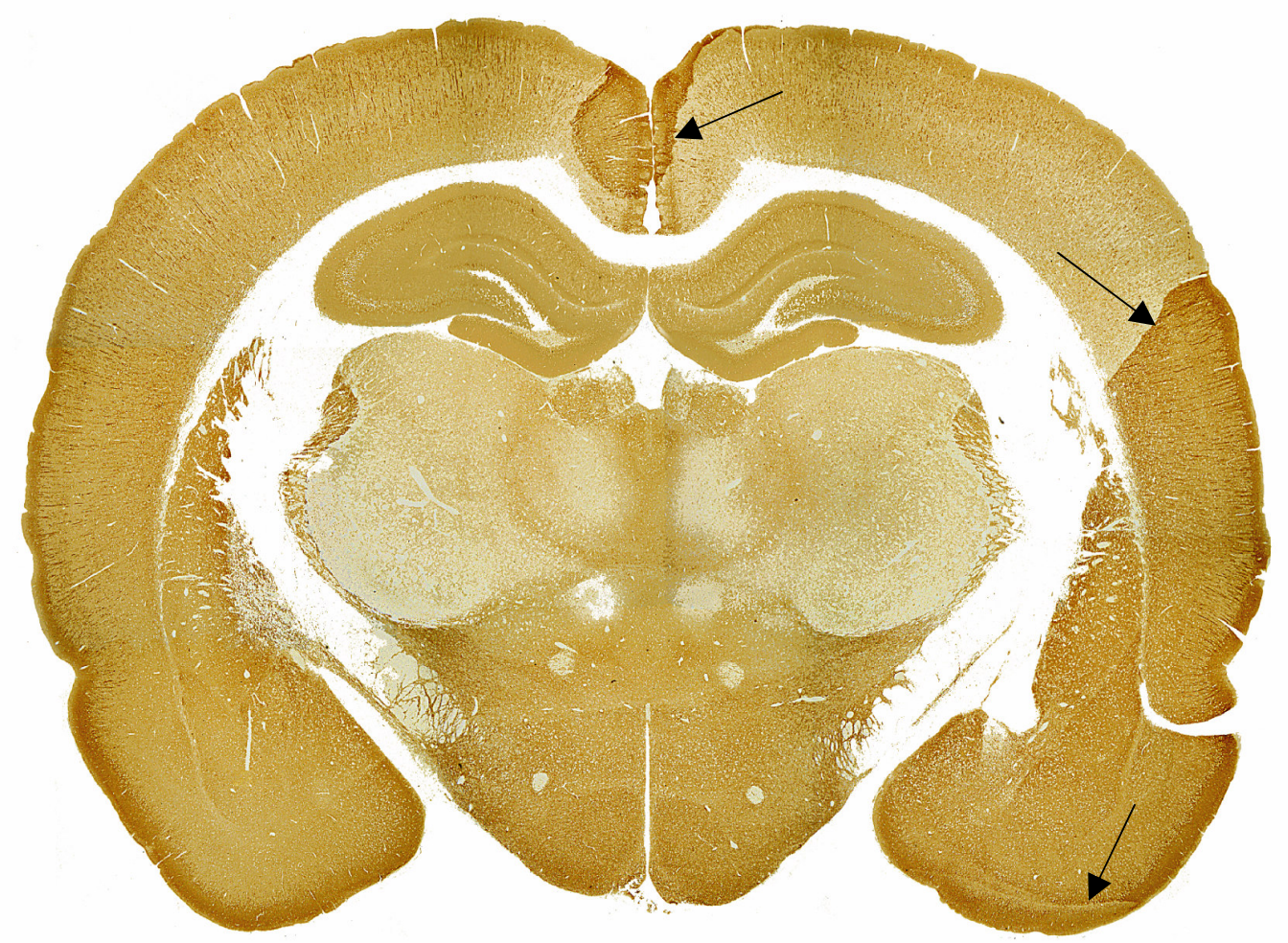

Figure 1. Whole brain section following microwave antigen retrieval at 98ºC, showing uneven patterns of MAP-2 staining. Darkened blotchy areas with adjacent light patches are visible (arrows), yielding uninterpretable MAP-2 immunoreactivity when imaging analysis is performed.

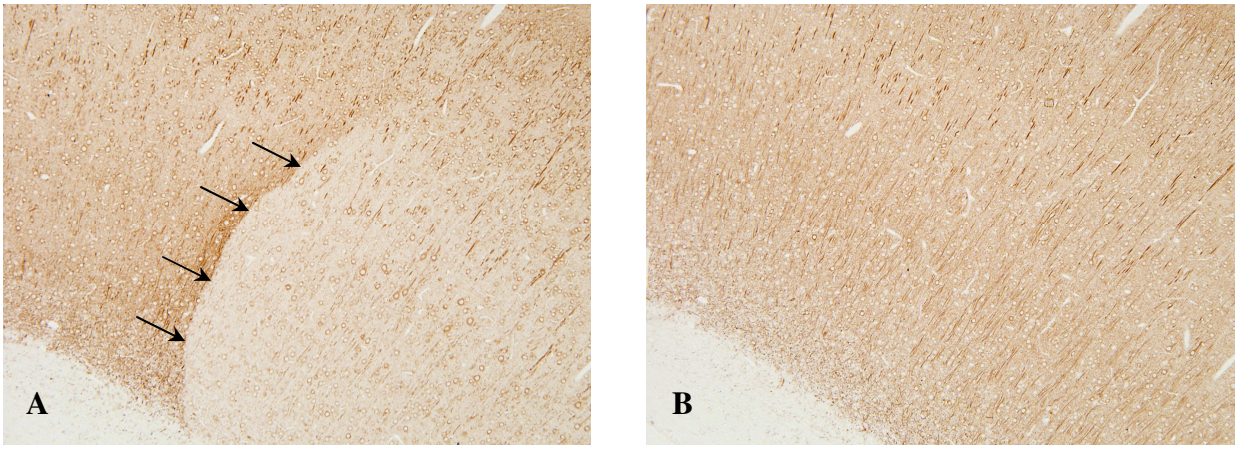

Figure 2. MAP-2 immunoreactivity in the cortex. Inconsistent distribution of MAP-2 staining (arrows) is seen following microwave antigen retrieval at 98℃ (A). Normal patterns of MAP-2 immunoreactivity are observed following microwave antigen retrieval at 106℃ (B). Magnification 10X.

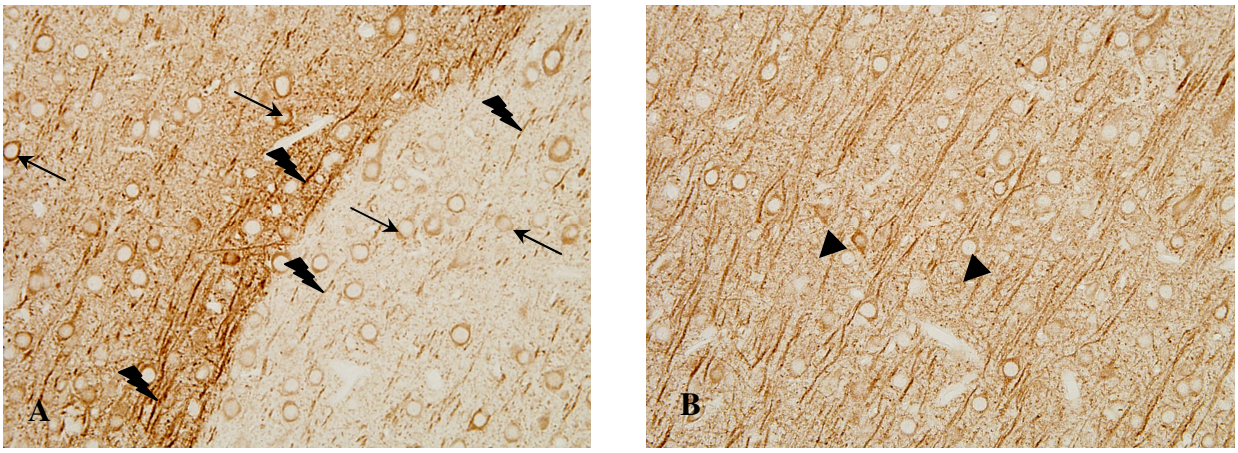

Figure 3. Higher magnification of MAP-2 immunoreactivity in the cortex. Following microwave antigen retrieval at 98℃ (A), dramatic variations in MAP-2 immunoreactivity are visible. Staining of neuronal soma (arrows) and apical dendrites (lightning bolts) is not of uniform intensity throughout the section. MAP-2 staining is enhanced following microwave antigen retrieval at 106℃ (B) and finer dendritic processes are visible (arrow heads). Magnification 20X.

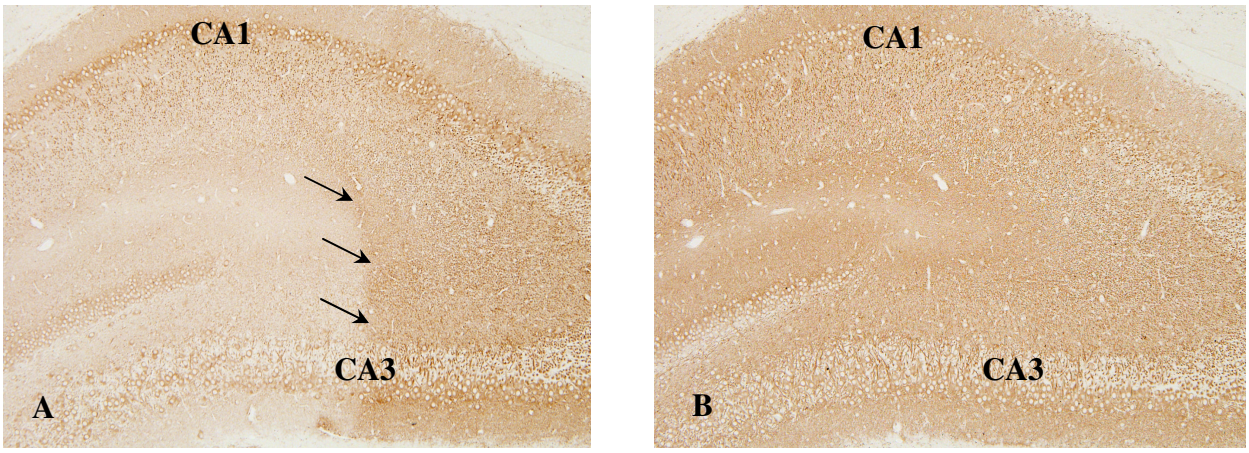

Figure 4. MAP-2 immunoreactivity in the hippocampus. MAP-2 staining is incrongruent (arrows) following microwave antigen retrieval at 98℃ (A). Even MAP-2 staining is seen after microwave antigen retrieval at 106℃ (B). CA1, CA1 subregion of the hippocampus; CA3, CA3 subregion of the hippocampus. Magnification 10X.

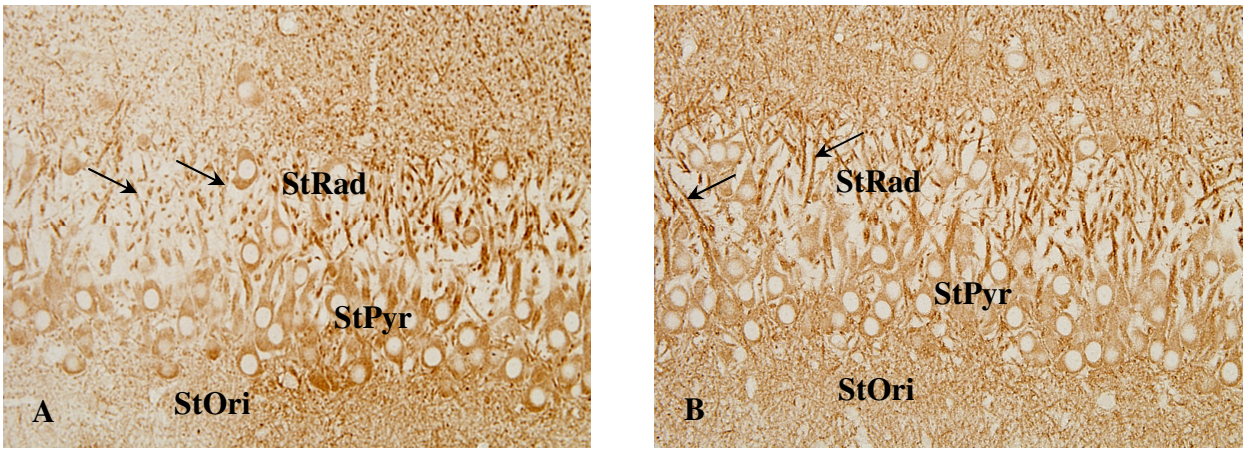

Figure 5. MAP-2 immunoreactivity in the CA3 region of the hippocampus. Following microwave antigen retrieval at 98℃ (A), MAP-2 staining of apical dendrites (arrows) of pyramidal neurons in the stratum radiatum appears fragmented. MAP-2 staining following microwave antigen retrieval at 106℃ is consistent and apical dendrites appear long and smooth (arrows). StRad, Stratum radiatum; StPyr, Stratum pyramidal; StOri, Stratum orien. Magnification 20X.

Conclusions

Previously, we established that the effectiveness of MAR is strongly influenced by the composition and pH of the AR solution and the specificity and avidity of the primary antibody (Pleva et al., 2002). However, high temperature heating of tissues is believed to be the critical factor of antigen retrieval (Shi et al., 1991). Our results indicate that temperature is crucial in recovering full staining with MAP-2 antibody. When sections were heated at 98°C, MAP-2 staining was inconsistent, but when sections were boiled at 106°C, markedly improved staining was observed. MAP-2 immunoreactivity appeared highly specific and evenly distributed throughout the brain section. Uniform patterns of MAP-2 staining are essential when evaluating dendritic pathology in formalin-fixed paraffin embedded brain sections. Inconsistent patterns of MAP-2 can be misinterpreted as a negative stain and, in addition, can hinder performing accurate quantitative analysis on sections.

Although the manufacturer of the EZ-Retriever™ microwave did not recommend setting the temperature parameter higher than 98°C with citra-based AR solutions, the importance of testing different temperatures for a particular AR solution is clear. When using the EZ- Retriever™ microwave, we conclude that boiling sections in 10 mM citric acid of pH 6.0 for two cycles of 5 min at 106°C is optimal for recovering MAP-2 antigenicity in formalin-fixed, paraffin-embedded sections. This standardization of the MAR process will undoubtedly aid in achieving consistent and reliable results in future immunohistochemical applications using MAP-2, as well as other proteins of interest.

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